

## Mammary Cell Number, Proliferation, and Apoptosis During a Bovine Lactation: Relation to Milk Production and Effect of bST<sup>1</sup>

A. V. Capuco,\* D. L. Wood,\* R. Baldwin,†  
K. Mcleod,† and M. J. Paape‡

\*Gene Evaluation and Mapping Laboratory,

†Nutrient Conservation and Metabolism Laboratory and

‡Immunology and Disease Resistance Laboratory

USDA-ARS, Beltsville, MD 20705

### ABSTRACT

This investigation evaluated mammary cell loss and replacement during lactation and the impact of administration of bST on these processes. During lactation, a gradual decrease in number of mammary epithelial cells within the mammary glands occurs and largely accounts for the decline in milk production with advancing lactation. This decrease is not appreciably impacted by the loss of viable epithelial cells in milk. Rather, the net decline in cell number (~50% during the entire lactation) results from continual death by apoptosis. Accompanying the decline in mammary cell number by apoptosis is a degree of cell renewal. Approximately 0.3% of mammary cells in lactating, nonpregnant cows were labeled by a 24-h in vivo treatment with the thymidine analog, bromodeoxyuridine. During the entire lactation, the number of new cells amounts to approximately 50% of the number of cells initially present. By the end of lactation, most cells present in the mammary gland were formed after calving. Increasing cell replacement or decreasing apoptosis during lactation may provide a means to increase persistency of lactation. Indeed, administration of bST to Holstein cows during midlactation increased the proportion of mammary epithelial cells expressing the nuclear proliferation antigen, Ki-67, from 0.5 to 1.6%. Bovine somatotropin appears to increase the rate of cell renewal in the lactating mammary gland. Knowledge of molecular regulation of apoptosis and cell proliferation should provide a means to modulate cell turnover in the mammary gland. A change in the ratio of epithelial proliferation to cell death during lactation will affect the persistency of lactation.

(**Key words:** bromodeoxyuridine, cell renewal, lactation curve, somatotropin)

**Abbreviation key:** BrdU = bromodeoxyuridine, PCA = perchloric acid, TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling.

### INTRODUCTION

Milk yield and the shape of the lactation curve are determined by the number of mammary secretory cells and the secretory activity per cell. Most mammary growth occurs during pregnancy, and in some species considerable growth occurs during early lactation (Anderson, 1974; Tucker, 1981). In dairy goats, mammary growth and differentiation during early lactation account for increasing milk yield during the ascending portion of the lactation curve, whereas after peak lactation, loss of mammary cells largely accounts for declining milk yield (Knight and Peaker, 1984). In contrast, declining milk yield during extended lactation in rats is primarily due to reduced secretory capacity per cell (Knight et al., 1984). For dairy cows, cell proliferation and death during lactation has not been evaluated, nor has the relationship between milk yield, cell number, and secretory activity per cell. Although a decline in mammary cell number during lactation must at least partially account for the decline in milk yield after peak (Capuco et al., 1997), it remains to be determined whether significant cell turnover occurs during lactation. Although there is little cell turnover during lactation in rats (Pitkow et al., 1972), the bovine lactation is markedly longer and may not be at all comparable with regard to cell kinetics.

During the past 40 yr, selective breeding has produced dramatic gains in the quantity of milk produced per dairy cow. This gain can be attributed to increased peak milk yield, without proportional increases in the persistency of lactation, i.e., reduction in slope of the declining phase of the lactation curve. However, if persistency of lactation could be increased, considerable benefits would accrue to the dairyman. By lengthening

Received December 15, 2000.

Accepted May 25, 2001.

Corresponding author: A. V. Capuco; e-mail: acapuco@anri.barc.usda.gov.

<sup>1</sup>Mention of a trade name or proprietary product does not constitute a guarantee or warranty by the USDA and does not imply approval to the exclusion of others not mentioned.

lactation, a smaller portion of the cow's life would be spent during the periparturient period with its increased health risks and associated costs, and reproductive efficiency could be increased by delayed breeding and by the potential use of sexed semen for AI in order to more closely match the herds needs for replacement heifers. Management schemes that improve maintenance of mammary cell number will certainly increase persistency. Indeed, although the mechanisms are uncertain, recent data indicate that bST increases persistency of lactation (Bauman et al., 1999; Van Amburgh et al., 1997).

The objectives of the present investigation were: 1) to determine whether the decline in milk production during advancing lactation is due to a decline in cell number or cell activity, 2) to evaluate cell proliferation and apoptosis during a bovine lactation, and 3) to assess the impact of bST on mammary cell proliferation and apoptosis during lactation.

## MATERIALS AND METHODS

### Cows and Experimental Design

**Experiment 1.** Twenty multiparous, nonpregnant, clinically normal, lactating Holstein cows from the dairy herd of the Beltsville Agricultural Research Center were used. Cows were housed in tie stalls with 12 h of light/d and were milked twice daily at 0700 and 0600 h. Cows were fed a TMR to provide 100% of NRC requirements daily at 0900 h. Feed consumption was monitored daily by weighing feed offered and orts. Four to six cows were slaughtered at each of four stages of lactation. These stages were 14 (four cows), 90 (five cows), 120 (six cows), and 240 d (5 cows) of lactation. Beginning 3 wk before slaughter, foremilk samples were collected aseptically once weekly to assess bacteriological status and SCC. All cows were free of clinical signs of mastitis and had four functional quarters. Five cows harbored subclinical infections in one or two quarters. Six quarters were subclinically infected with coagulase-negative staphylococci, and one quarter was subclinically infected with *Staphylococcus aureus*. SCC of subclinically infected quarters was  $<500 \times 10^3$ . Average SCC for all quarters of all cows was 52,000 cells/ml (two samples before slaughter for 14 d group and three weekly samples for all other groups).

All cows, except for those at 14 d of lactation, were synchronized with two injections of prostaglandin  $F_{2\alpha}$  (Lutalyse, The Upjohn Co., Kalamazoo, MI) 14 d apart. Cows were killed 14 d after the second injection during the luteal phase. Before slaughter, each cow received four injections of bromodeoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) during a 24-h period, via indwelling jugular catheter. BrdU was administered

at a dosage of 2.25 mg/kg of BW per injection at a concentration of 20 mg/ml of 0.9% saline, pH 8.2. Dose of BrdU was based upon previous dose response data (Yanai et al., 1996). Injections were 7.5 h apart so that time between first and last BrdU injections was 22.5 h. Cows were killed 1 to 2 h after the final injection of BrdU. This is subsequently referred to as a 24-h BrdU treatment period.

**Experiment 2.** Effects of bST administration on cell proliferation and programmed cell death (apoptosis) were evaluated in lactating mammary tissues from cows that were treated with bST under conditions of differing energy balance. Cows and experimental protocol were the same as those described previously (Capuco et al., 2001). Twelve primiparous Holstein cows were paired based on milk production, and randomly assigned to be fed ad libitum or a restricted intake (80% of their ad libitum intake). Cows in each dietary intake group were randomly assigned to receive daily subcutaneous injections of bST (sometribove, 40 mg/d; provided by Monsanto Co., St. Louis, MO) or vehicle for 7-d periods according to a crossover design. There was a 14-d ad libitum feeding period before initiation of treatment and between treatment periods. Thus, all cows underwent a bST and a control period. Cows were housed in tie stalls with 12 h of light/d and were milked twice daily.

Use of animals for these investigations was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

### Tissue Samples

**Experiment 1.** At slaughter, the udder was removed, trimmed of extraparenchymal tissue, and separated into right and left halves. Each udder half was weighed and parenchyma samples were collected from left front and rear quarters. However, right quarters of three cows were sampled because subclinical infections with elevated SCC were found in left quarters. Tissue was placed in Medium 199 (Gibco BRL, Grand Island, NY) and transported to the laboratory for incubation with  $^3\text{H}$ -thymidine. Tissue samples for quantitation of DNA and RNA were frozen immediately in liquid nitrogen and stored at  $-80^\circ\text{C}$  until assay. Mammary tissue samples for immunohistochemistry were fixed overnight in 10% neutral buffered formalin at  $4^\circ\text{C}$  and then stored in 70% ethanol until further processing. Tissues were then dehydrated and embedded in paraffin according to standard techniques and sectioned at  $6\text{-}\mu\text{m}$  onto silanated slides.

**Experiment 2.** Mammary biopsies were obtained on the last day (d 7) of each treatment period. Biopsies were taken from rear quarters using a tool designed to

obtain a core of mammary parenchyma (Farr et al., 1996). Tissues were fixed in 10% neutral buffered formalin overnight at 4°C and transferred to 70% ethanol until further processing. Tissues were then dehydrated and embedded in paraffin according to standard techniques and sectioned at 6- $\mu$ m onto silanated slides.

### Immunohistochemistry

**BrdU and Ki-67 localization.** Slides were deparaffinized in xylene and hydrated in a graded series of ethanol. Tissue sections were quenched with 3% H<sub>2</sub>O<sub>2</sub> in Dulbecco's PBS (pH 7.4) for 10 min and then washed in PBS (3  $\times$  2 min). Microwave antigen retrieval was then used (Shi et al., 1991). Slides were heated in a microwave at high power (650W) in 400 ml of 10 mM citrate buffer (pH 6.0) in a covered glass staining dish for 5 min, remained undisturbed for 5 min, and then were microwaved for an additional 5 min. Slides remained in the buffer for a 30-min cooling period. They were then washed in PBS (3  $\times$  2 min) and blocked with 5% nonimmune goat serum in PBS (30 min) before histochemical localization of BrdU or Ki-67 antigen.

Immunolocalization of BrdU-labeled cells was essentially as described previously (Garrett and Guthrie, 1998). After blocking with nonimmune goat serum, slides were incubated with BrdU monoclonal antibody (clone BMC 9318, Boehringer Mannheim) overnight at 4°C. Antibody concentration was 2  $\mu$ g/ml in PBS containing 1% normal goat serum. Slides were then washed in PBS (3  $\times$  5 min). A 1:200 dilution of goat anti-mouse IgG (Sternberger Monoclonals Inc. Lutherville, MD) was prepared in PBS containing 1% normal goat serum and 2% normal calf serum and incubated for 30 min at room temperature. Slides were then incubated with the adsorbed second antibody for 30 min at room temperature. They were washed in PBS (3  $\times$  5 min) and then incubated with mouse peroxidase-anti-peroxidase (mouse clonoPAP, Sternberger Monoclonals, Inc.), at a dilution of 1:200 in PBS containing 1% normal goat serum, for 30 min at room temperature. After washing in PBS (3  $\times$  5 min), sections were incubated with the chromagen, diaminobenzidine. Slides were washed with distilled H<sub>2</sub>O, and tissue sections were counterstained with hematoxylin, dehydrated, and mounted with Permaslip (Alban Scientific Inc., St. Louis, MO).

After quenching with H<sub>2</sub>O<sub>2</sub>, microwave antigen retrieval and blocking with 5% nonimmune goat serum as described above, staining Ki-67 antigen utilized the MIB-1 monoclonal antibody (prediluted MIB-1, Zymed Laboratories, San Francisco, CA) and Histostain SP kit (Zymed). The slides were incubated with primary antibody for 60 min at room temperature and then washed in PBS (3  $\times$  5 min). Slides were then incubated

for 10 min at room temperature with biotinylated second antibody and washed in PBS (3  $\times$  2 min). Slides were then incubated with streptavidin-peroxidase-conjugate for 10 min at room temperature. After washing in PBS (3  $\times$  5 min), sections were incubated with diaminobenzidine. Slides were washed with distilled H<sub>2</sub>O, sections counterstained with hematoxylin, dehydrated, and then mounted with Permaslip.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling.** In situ detection of apoptotic cells utilized terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to visualize cells that exhibited endonucleolytic degradation of DNA, a key feature of apoptotic cells. A commercial kit (Apotag kit, Oncor, Gaithersburg, MD) was used. After deparaffinization and hydration, slides were incubated with proteinase K (20  $\mu$ g/ml of PBS, Oncor). The manufacturer's recommended protocol was used. Sections were counterstained with methyl green and mounted with Permaslip.

**Quantitation of immunohistochemistry.** Tissue sections were viewed by light microscopy to quantify BrdU-labeled cells, Ki-67 antigen expressing cells, and apoptotic cells. For each tissue section, 10 microscopic fields were quantified. A field was selected under low power and slightly out of focus, then the objective was switched to higher power and the cells within an 8  $\times$  8 grid were counted at 500 $\times$  magnification. For experiment 1, a single section from the left rear and one from the left front quarters were evaluated per cow. At least 1500 cells were counted per section, 3000 cells per cow. For experiment 2, one section per cow was evaluated during the control and the bST treatment periods. At least 1500 cells were evaluated per section.

### <sup>3</sup>H-Thymidine Incorporation

Mammary tissue from cows in experiment 1 was sliced with a Stadie-Riggs hand microtome (Arthur H. Thomas Co., Philadelphia, PA). Triplicate slices (~150 mg) from front and rear glands were each incubated in 3 ml of Medium 199 containing 1  $\mu$ Ci of <sup>3</sup>H-thymidine/ml. Incubation was for 2 h at 37°C in a shaking water bath, under an atmosphere of 5% CO<sub>2</sub> and 95% O<sub>2</sub>. After incubation, tissues were rinsed in 0.9% saline and frozen at -20°C until quantification of incorporation. Tissue slices were homogenized in saline, precipitated with trichloroacetic acid, and radioactivity determined by liquid scintillation spectroscopy, as described previously (Capuco and Akers, 1990).

### DNA and RNA Analyses

To quantify nucleic acids, mammary tissue was homogenized (1:15 wt/vol) in DNA assay buffer (50 mM



Na<sub>2</sub>PO<sub>4</sub>, 2 M NaCl, 2 mM Na<sub>2</sub>EDTA) with a Tekmar Homogenizer (Tekmar, Cincinnati, OH). DNA was quantified using the Hoechst 33258 dye binding (LaBarca and Paigen, 1980). Five-microliter aliquots of sample homogenates were transferred to a 96-well microplate. Then 195  $\mu$ l of DNA assay buffer containing Hoechst dye [99.8 ml of DNA assay buffer + 200  $\mu$ l of dye solution (1  $\mu$ g of Hoechst 33258 dye/ $\mu$ l of distilled water)] was added to each sample. A standard curve containing 0 to 2  $\mu$ g of calf thymus DNA per well was prepared. Fluorescence was read with a Bio-Tek FL600 plate reader with 360/460 nm filter set (Bio-Tek Instruments, Inc., Winooski, VT).

RNA was determined by ultraviolet absorbance. A 0.5-ml aliquot of the above mammary homogenate was diluted with an equal volume of phosphate buffer to which 0.33 ml of ice-cold 1.2 N perchloric acid (PCA) was added, and tubes were kept on ice for 10 min. After centrifugation at 3600  $\times$  g for 15 min at 4 C, the supernatant was discarded. The pellet was washed by resuspension in 0.75 ml of 0.2 N PCA and centrifugation at 3600  $\times$  g for 15 min at 4°C. The pellet was washed two additional times with 0.4 ml of 0.2 N PCA; after which it was suspended in 1 ml of 0.3 N PCA and incubated at 37° for 60 min, with periodic vortexing. Tubes were then incubated on ice for 10 min after the addition of 0.67 ml of ice-cold 1.2 N PCA. Samples were centrifuged at 3600  $\times$  g for 15 min at 4°C and supernatant collected. The pellet was washed 3 times with 0.34 ml of 0.2 N PCA, and all supernatants were combined. A portion of the collected supernatants was diluted, and absorbance was measured at 260 and 232 nm with a Beckman DU 650 (Beckman Instruments, Inc., Fullerton, CA).

## Bacteriology and SCC

Foremilk samples were collected aseptically for bacteriological analysis and to determine milk SCC. Teats were cleaned with ethanol and milk samples for bacteriology were collected midstream during the second expulsion of milk. Approximately 20 ml of foremilk was then collected for determination of SCC. For experiment 1, foremilk samples were obtained periodically throughout the study. Three samples were obtained during the final 2 wk and the final sample on the day of slaughter. In experiment 2, foremilk samples were obtained at the start of the experiment and on the time of each biopsy. Milk samples were cultured on tryptose-blood agar plates containing washed bovine red blood cells and esculin. Plates were incubated at 37°C for 48 h and examined to determine their bacteriological status (National Mastitis Council, 1981). Milk SCC of each sample was determined with a Fossomatic 90 somatic cell

counter (Foss Electric, Hillerød, Denmark). The cell counter was calibrated monthly with bovine milk SCC standards (Dairy Quality Control Institute Services, Mountain View, MN). Duplicate counts were made on each milk sample.

## Growth Curves

Curves predicting the DNA content during lactation and the accumulated cell proliferation and cell death were generated based upon daily rates of cell proliferation and death estimated in experiment 1. The DNA content or cell number (N) on a given day is described by the following exponential equation, where N<sub>0</sub> = cell number on d 0:

$$N = N_0 * e^{(\text{daily rate of proliferation} - \text{daily rate of cell death}) * \text{day}}$$

This can also be closely approximated by the following equation, where R = 1 + (daily rate of proliferation – daily rate of death) and N<sub>0</sub> = cell number on d 0:

$$N = N_0 * R^{\text{day}}$$

The accumulated number of cells (or DNA) that have proliferated until a given day is described by the following collection function, where P<sub>0</sub> = N<sub>0</sub> \* daily rate of proliferation:

$$\text{Accumulated Cell Proliferation} = \sum_0^{\text{day}} P_0 * R^{\text{day}}$$

Similarly, the accumulated loss of cells (or DNA) until a given day is described by the following collection function, where L<sub>0</sub> = N<sub>0</sub> \* daily rate of cell death:

$$\text{Accumulated Cell Loss} = \sum_0^{\text{day}} L_0 * R^{\text{day}}$$

## Statistical Analyses

Data in experiment 1 were analyzed using a one-way ANOVA. Data in experiment 2 were analyzed using a two-way ANOVA with repeated measures. Bonferroni's multiple comparison test was used for post ANOVA comparisons (Prism, version 3; GraphPad Software, Inc., San Diego, CA).

## RESULTS AND DISCUSSION

### Experiment 1

Multiparous Holstein cows used in experiment 1 averaged 11,876 kg of milk during lactation (305 d) and

**Table 1.** Relationship between stage of lactation, total mammary and epithelial cell number, and secretory activity per cell.

	Day of Lactation				SE <sup>1</sup>
	14	90	120	240	
N	4	5	6	5	
Body weight, kg <sup>2</sup>	727 <sup>a</sup>	628 <sup>a,b</sup>	572 <sup>b</sup>	706 <sup>a</sup>	37
Lactation number	4.0 <sup>a</sup>	2.6 <sup>a</sup>	3.33 <sup>a</sup>	3.6 <sup>a</sup>	0.81
Lactation yield, kg <sup>3</sup>	11,612 <sup>a</sup>	11,267 <sup>a</sup>	11,381 <sup>a</sup>	12,977 <sup>a</sup>	1395
Milk, kg/d <sup>4</sup>	39.9 <sup>a,b</sup>	47.3 <sup>a</sup>	44.6 <sup>a</sup>	36.5 <sup>b</sup>	2.1
Parenchyma, kg <sup>5</sup>	27.3 <sup>a</sup>	24.2 <sup>a</sup>	23.3 <sup>a</sup>	23.1 <sup>a</sup>	1.7
Total DNA, g <sup>5</sup>	96.4 <sup>a</sup>	68.9 <sup>b</sup>	64.1 <sup>b</sup>	58.1 <sup>b</sup>	6.7
RNA/DNA	1.14 <sup>b</sup>	1.69 <sup>a,b</sup>	1.35 <sup>b</sup>	2.05 <sup>a</sup>	0.15
% Epithelium	72.9 <sup>b</sup>	79.4 <sup>a</sup>	73.6 <sup>b</sup>	72.8 <sup>b</sup>	1.5
Epithelial DNA, g <sup>5,6</sup>	70.5 <sup>a</sup>	52.9 <sup>b</sup>	50.7 <sup>b</sup>	42.2 <sup>b</sup>	5.2
Milk, kg/d * g total DNA <sup>5</sup>	0.42 <sup>b</sup>	0.74 <sup>a</sup>	0.67 <sup>a</sup>	0.66 <sup>a,b</sup>	0.06
Milk, kg/d * g epithelial DNA <sup>5</sup>	0.58 <sup>b</sup>	0.93 <sup>a</sup>	0.90 <sup>a</sup>	0.90 <sup>a</sup>	0.19

<sup>a,b,c</sup>Means without a common superscript differ ( $P < 0.05$ ).

<sup>1</sup>SE appropriate for  $n = 4$ .

<sup>2</sup>Body weight per cow was the average of weights on the day of slaughter and 2 to 4 d earlier.

<sup>3</sup>Milk production (305 d) for current lactation, or previous lactation of the 14-d group.

<sup>4</sup>Average milk yield for 7 d preceding slaughter.

<sup>5</sup>Measures for the entire udder.

<sup>6</sup>Estimated for each cow as the total DNA times the average epithelial percentage for the group.

were on average in their third lactation (Table 1). Cows that were slaughtered at 14, 90, 120, or 240 d of lactation did not differ with regard to production level or lactation number ( $P > 0.05$ ). As expected, daily milk yield was greatest at 90 d and was lowest at 240 d ( $P < 0.05$ ). Total number of mammary cells, i.e., total parenchymal DNA, was greatest at 14 d of lactation and declined to a low at 240 d ( $P < 0.05$ ). The RNA/DNA ratio was not positively correlated with milk yield and was greatest at 240 d of lactation ( $P < 0.05$ ). Although unexplained, a similar increase in RNA/DNA was observed during late lactation in goats (Knight and Peaker, 1984). During early lactation (14 d), mammary cell number was at its peak, whereas milk yield per cell and RNA/DNA ratio were at their lowest ( $P < 0.05$ ). Thereafter, milk yield per unit of mammary DNA remained constant during lactation ( $P > 0.05$ ). The proportion of epithelial cells in mammary tissue was influenced by stage of lactation ( $P = 0.008$ ). Quantitative histological analysis showed that the proportion of epithelial cells was low at 14 d, peaked at 90 d of lactation and then declined. Mean epithelial percentages at each stage of lactation were used to estimate DNA of epithelial origin. Number of epithelial cells (epithelial DNA) was greatest during early lactation and declined with advancing lactation ( $P = 0.007$ ). Milk yield per epithelial cell increased from early to peak lactation ( $P < 0.05$ ) and then remained constant with advancing lactation ( $P > 0.05$ ).

The increase in milk yield until peak lactation appeared due to continued differentiation of the mam-

mary epithelium, rather than an increase in number of secretory cells, whereas the decline in milk production after peak lactation was attributable to loss in number of secretory cells, rather than loss in secretory activity per cell. Milk yield declined 23% from peak production at 90 d, and mammary DNA (total or epithelial) declined 17% from 90 to 240 d, and 40% from peak parenchymal DNA at 14 d to a low at 240 d. These data are in general agreement with the demonstration that the primary factor contributing to declining milk yield during lactation in goats is a decrease in mammary cell number (Knight and Peaker, 1984). It is important to realize that the current data pertain to nonpregnant multiparous cows. When cows are concomitantly lactating and pregnant, it is likely that a decline in secretory capacity per mammary cell accompanies advanced pregnancy, due to the conflicting metabolic demands of gestation and lactation. Indeed, this is readily apparent during late pregnancy, when the number of mammary epithelial cells actually increases simultaneous with a rapid decline in milk production (Capuco et al., 1997).

To evaluate changes in the size of a cell population and the extent of cell turnover in that population, it is necessary to quantify rates of cell proliferation and cell death. Cell number is a function of the *relative* rates of cell proliferation and cell death. The mammary gland grows when the rate of proliferation exceeds the rate of cell death, and it regresses when the rate of cell death exceeds the rate of cell proliferation. When the rates of proliferation and death are equal, the mammary gland remains in a steady state with respect to cell number.

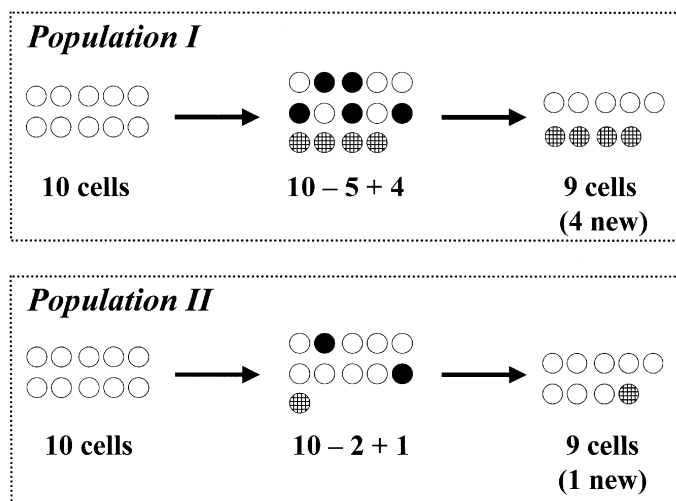
Regardless of the net change in cell number, a population may undergo varying degrees of cell replacement, or turnover (Figure 1). The extent of cell turnover is determined by the *absolute* rates of proliferation and cell death, processes that are very difficult to quantify *in vivo*. Most often, the relative states (not rates) of proliferation and death are assessed. As illustrated in Figure 1, the same net change in cell number can be realized with markedly different rates of cell renewal.

In experiment 1, rates of mammary cell proliferation at four stages of lactation were determined by injecting the thymidine analog, BrdU, at 7.5-h intervals for 23 h and quantifying labeled cells by immunohistochemistry. Assuming the S-phase of the cell cycle is 7.5 h or more, this injection protocol should label all cells that synthesize DNA within the 24-h labeling period. This seems a fair assumption because the duration of S-phase in rodent mammary epithelial cells ranges from 8.2 to 14.1 h, depending upon physiological state (Banerjee and Walker, 1967). Additionally, a 24-h labeling period provides the means to obtain a reliable measure of average proliferation rate even in the presence of an underlying diurnal pattern of DNA synthesis, such as that reported for rodent mammary gland (Borst and Mahoney, 1980). Two additional methods for assessing relative states of cell proliferation were employed in the present study: *in vitro* incorporation of  $^3\text{H}$ -thymidine by

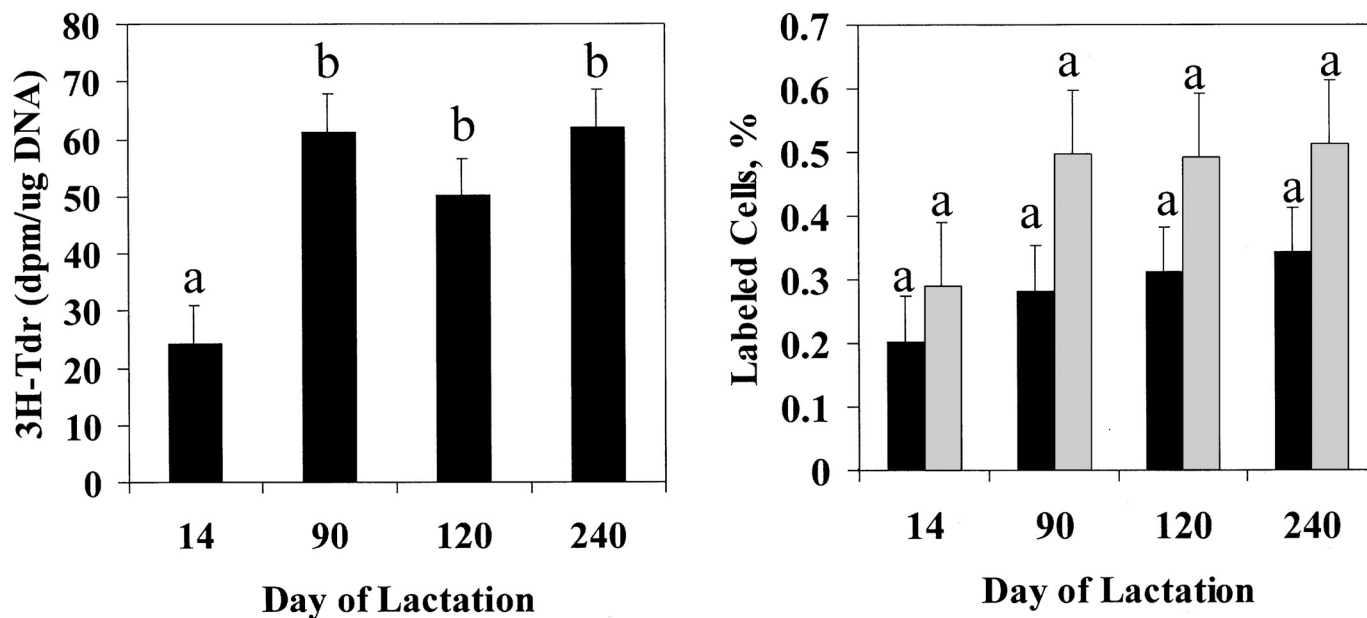
mammary tissue slices and histochemical quantification of cells expressing the Ki-67 nuclear proliferation antigen. The Ki-67 nuclear proliferation antigen is expressed during all phases of the cell cycle except  $G_0$ , and provides an estimate of the tissue growth fraction (Gerdes et al., 1984, 1991). Although the function of this conserved protein is unknown, it is tightly associated with chromatin, and correlates with the proliferative status of tissues under a variety of physiological and pathological conditions (Gerdes et al., 1991; Sakaguchi et al., 2000; Scholzen and Gerdes, 2000; Shayan et al., 1999). Of the three methods used to assess cell proliferation, only the BrdU labeling scheme as employed provides an estimate of the cellular *proliferation rate*.

The proliferative status of mammary tissue at four stages of lactation is summarized in Figure 2. The three methods of assessing the relative degree of cell proliferation were in general agreement. When rates of DNA synthesis were assessed by incorporation of  $^3\text{H}$ -thymidine, cell proliferation was lower ( $P < 0.05$ ) during early lactation (14 d) than during later stages of lactation. Although the number of BrdU-labeled cells and Ki-67 expressing cells were not influenced by stage of lactation ( $P > 0.05$ ), the arithmetic means were similarly lower during early lactation than during later stages of lactation. These data, in conjunction with a higher apoptotic index during early lactation (Figure 3), suggest that significant mammary growth does not extend from pregnancy into early lactation, as occurs in a number of species (Anderson, 1974, 1975; Anderson et al., 1981). Thus, the increase in milk production from parturition to peak lactation can be attributed to increased synthetic capacity per cell (hypertrophy) rather than increased cell proliferation (hyperplasia). The latter conclusion is somewhat tentative because mammary growth during lactation may have occurred before the first sampling time, 14 d of lactation, in the current study. On the other hand, some growth of caprine mammary gland continues until 21 d of lactation.

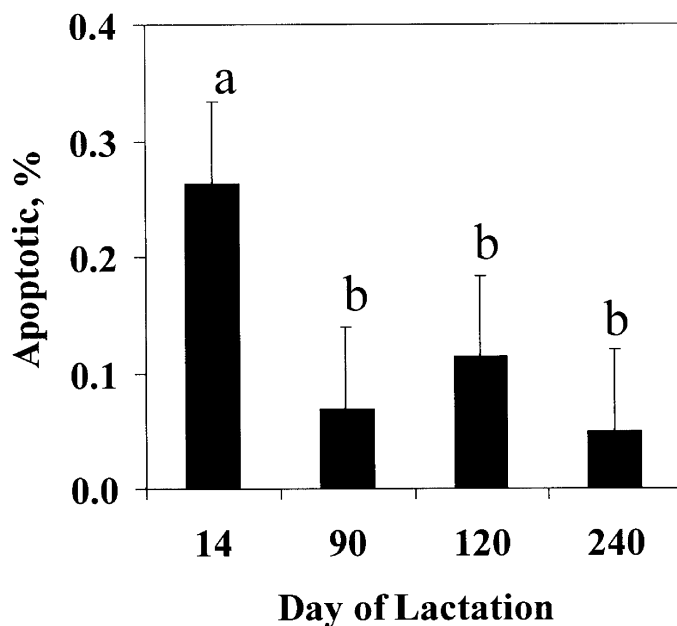
The apparent lack of mammary growth during early lactation in the present study does not preclude the mammary gland's ability to respond to different management conditions. For example, milking six times daily during early lactation caused an increase in milk yield that persisted after normal milking was resumed (Bar-Peled et al., 1995). Although not demonstrated, this carryover effect is consistent with stimulation of mammary growth during early lactation. Finally, net mammary growth can occur during lactation, as demonstrated by the compensatory growth response to various stimuli (Capuco and Akers, 1990; Knight and Peaker, 1984). Suckling and milk removal during early lactation are major controlling factors of mammary cell number



**Figure 1.** Rates of cell proliferation and cell death determine the net gain or loss of cells and extent of cell renewal within the population. Depicted is a population of cells undergoing net regression. Open circles represent the initial populations of cells, new cells formed by cell proliferation are depicted by cross-hatched circles, and cells that die during this period are depicted by black circles. In both panels, the net loss is identical because the difference between rates of cell death and proliferation are one cell. However, cell renewal differs markedly between panels. In the upper panel, the population of nine cells contains four new cells. In the lower panel, the population of nine cells contains one new cell.



**Figure 2.** Mammary cell proliferation during lactation. Left panel: Incorporation of  $^3\text{H}$ -thymidine during a 2-h incubation of mammary tissue slices. Right panel: Bromodeoxyuridine (black bars) and Ki-67 (gray bars) labeling index. Data are expressed as a percentage of total cells. Each bar represents the mean  $\pm$  SE for four to six cows. Within each category of assessing proliferation, means without a common superscript differ ( $P < 0.05$ ).



**Figure 3.** Mammary cell apoptotic index during lactation. Data are expressed as a percentage of total cells. Each bar represents the mean  $\pm$  SE for four to six cows. Apoptotic index was greatest during early lactation ( $P < 0.05$ ), but did not vary after peak lactation.

in rodents. It remains to be demonstrated whether similar mechanisms are operative in dairy cows.

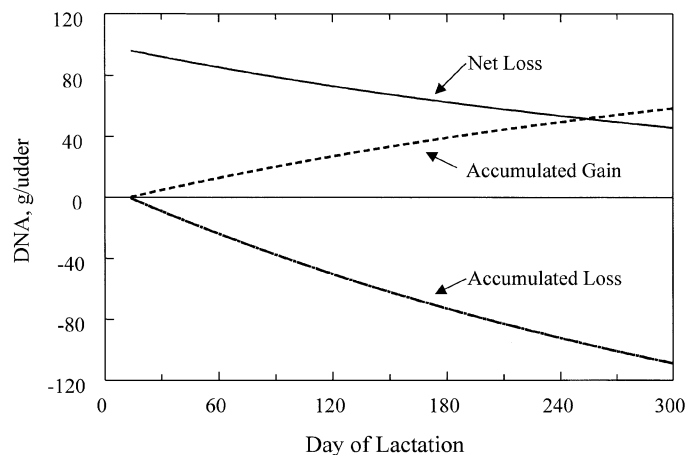
Considerable mammary cell turnover occurred during lactation, although the tissue was characterized by low indices of BrdU labeling and apoptosis. Approximately 0.3% of total mammary cells proliferated within a 24-h period, a proliferation rate that was unaffected by stage of lactation ( $P > 0.05$ , Figure 2). On the other hand, the apoptotic index was influenced by stage of lactation ( $P < 0.05$ , Figure 3). At 14 d, the apoptotic index was 0.27% of total cells, whereas for the remainder of lactation, it averaged 0.07% ( $P > 0.05$ ). The greater apoptotic index at 14 d of lactation likely reflects an increase in apoptotic leukocytes in the mammary gland and increased tissue edema that was evident at this time. During early lactation, migration of leukocytes into the mammary gland is enhanced, resulting in increased concentrations in milk and in mammary tissue (Concha, 1986; Sordillo et al., 1997). The high proportion of apoptotic cells noted in the mammary stroma at this time is consistent with an abundance of apoptotic leukocytes in the stroma. Because morphological features that distinguish cell types are typically lost during advanced stages of apoptosis such as those detected by the TUNEL, we cannot identify the cells in



the mammary stroma that underwent apoptosis. We suggest that the incidence of apoptosis among mammary cells remains relatively constant during lactation, and the increased incidence at 14 d was due to tissue edema and increased apoptotic leukocytes. Although an average apoptotic index of 0.07% provides an initial impression that cell proliferation exceeds cell death, it must be appreciated that this value is the proportion of apoptotic cells at a given moment and not the apoptotic rate. It has been estimated that the duration of apoptosis is approximately 3 h (Bursch et al., 1990). Therefore, the apoptotic rate can be estimated as being 0.56% of mammary cells per 24 h ( $0.07\% \times 24\text{h}/3\text{h}$ ) and clearly exceeds the measured proliferation rate of 0.3% of mammary cells per 24 h. The net result is a gradual loss of mammary cells during the course of lactation.

Another potential route for declining cell numbers during lactation is the continuous loss of mammary epithelial cells in milk. In the present experiment, milk SCC averaged 52,000 cells/ml, and milk production averaged 9920 kg for 240 d (calculated as 84% of 305-d DHI milk yield; personal communication, Paul Van Raden, USDA-ARS). Because epithelial cells account for less than 20% of milk SCC (Miller et al., 1991) and DNA content is 6 pg/cell, accumulative loss of mammary DNA is less than 0.62 g by 240 d of lactation. This accounts for only 1.6% of the net loss of mammary DNA (38.3 g) by 240 d of lactation. Although clearance of apoptotic cells from the mammary gland may involve passage of phagocytes into the milk, removal of intact viable mammary cells via the milk does not significantly contribute to the declining number of mammary secretory cells during lactation.

Despite the apparent low rate of mammary cell proliferation, there is considerable cell turnover during a bovine lactation. Applying the estimated apoptotic rate of 0.56% and a proliferation rate of 0.3% per 24 h, curves were generated for the accumulative cell loss, cell proliferation, and net cell loss (Figure 4). The net change in mammary cell number predicted from these fitted curves agrees reasonably well with the observed data. At 240 d of lactation, average mammary DNA was 58.1 g, and the predicted quantity was 53.3 g. At that time, the total quantity of DNA lost by apoptosis was 92 g, and the total DNA synthesized was 49.3 g. If none of the cells that died by apoptosis were those that proliferated during lactation, then 92% of the mammary cells remaining ( $49.3/53.3$ ) at 240 d of lactation were formed during lactation. If apoptotic cell death is random, then turnover of mammary cells will be less than 92% but certainly greater than 50%. The number of cells formed during lactation is predicted to equal the number that are present in the gland at 252 d of lactation (Figure 4).



**Figure 4.** Mammary DNA (cell) turnover during lactation. The proliferation rate was 0.003/d. The apoptotic index averaged 0.0007. Assuming a 3-h duration of apoptosis, the apoptotic rate is 0.0056/d. The accumulated synthesis of new DNA (formation of new cells) is indicated by the dotted line and described by the function:  $accumulated\ DNA\ gain = \sum_{day=0}^{day} 0.2892 * 0.9974^{day}$ . The accumulated loss of DNA by apoptosis is indicated by the dashed-dotted line and described by the function:  $accumulated\ DNA\ loss = \sum_{day=0}^{day} 0.5398 * 0.9974^{day}$ . Net loss of DNA (difference between synthesis and loss) is shown by the solid line and described by the equation:  $DNA = 96.4 * 0.9974^{day}$ .

The extent of mammary cell turnover during a bovine lactation differs markedly from that during a murine lactation. In contrast to the extensive cell turnover during a bovine lactation, approximately 75% of mammary cells are maintained throughout an entire lactation in rats (Pitkow et al., 1972). This lack of cell turnover may be consistent with the decline in activity per mammary secretory cell that occurs during lactation in rats but not in dairy cows.

Our estimation of the extent of apoptosis during lactation differs greatly from that of Wilde and colleagues (Wilde et al., 1997). In their investigation, the apoptotic index for mammary cells of Friesian-Holstein cows at 217 to 252 d of lactation averaged 2.4%. This value is approximately 35-fold greater than the proportion of apoptotic cells observed in the current study. Applying our earlier assumptions about the duration of apoptosis, this figure would equate to an apoptotic rate of 19.2% per day. In the absence of a proportional increase in proliferation rate (i.e., assuming the same proliferation rate as determined in the current study), this would result in a 97% decline in mammary cell number within 2 wk. Clearly, this is incompatible with maintenance of lactation. However, Wilde et al. (1997) did not indicate the reproductive status of cows used in their study. Because preliminary investigations (A. V. Capuco, unpublished) suggest that pregnancy in-

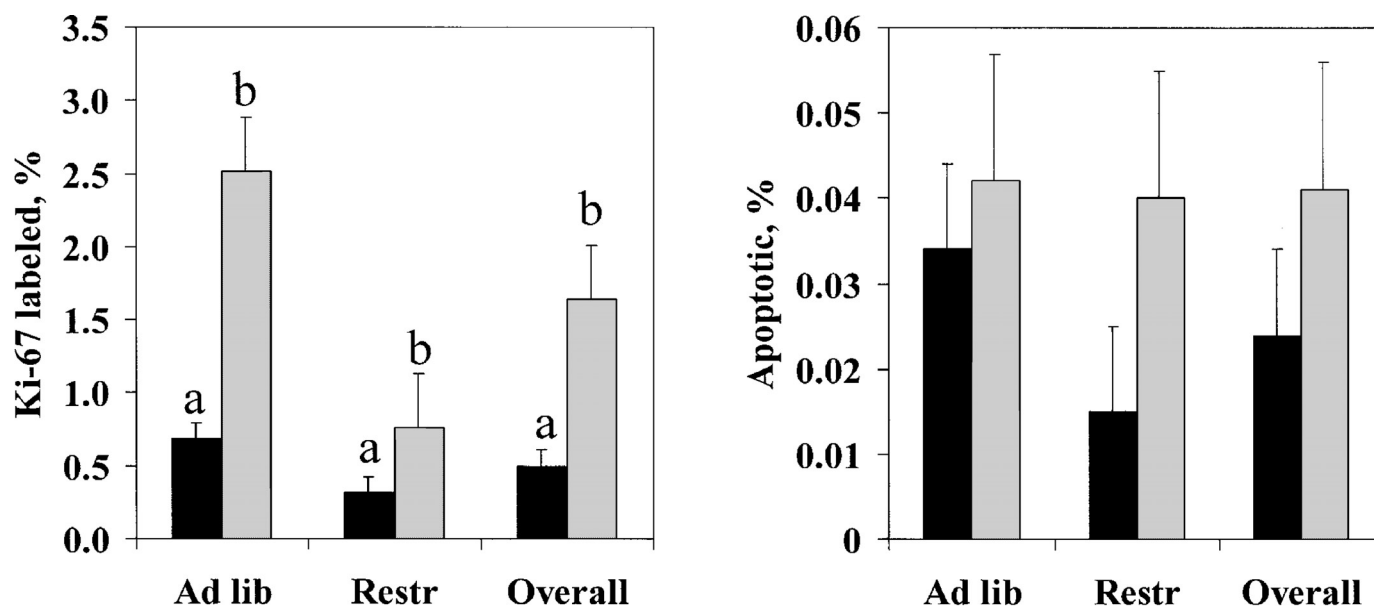


creases the rates of mammary cell apoptosis and proliferation in lactating cows, a difference in pregnancy status could account for the discrepancy between the two studies. The influence of pregnancy on mammary cell kinetics requires additional study.

After peak lactation, there was a decline in the number of mammary epithelial cells in the udder (Table 1). Expressed as a percentage of total cells, the epithelial compartment declined from 79% at 90 d to 73% at 240 d. This is consistent with previous data indicating that around the time of calving, 83% of mammary cells were epithelial, but during late lactation the percentage had decreased to 74% (Capuco et al., 1997). Apparently, because of the error inherent in quantifying very small percentages of proliferating and apoptotic cells, we were unable to subdivide the predictive equations in Figure 4 to adequately describe changes in the epithelial and stromal compartments.

The influence of bST on the proliferative and apoptotic status of cells within the lactating mammary gland was also evaluated. Previous experiments had indicated that bST increased the persistency of lactation (Bauman et al., 1999; Van Amburgh et al., 1997). In light of our current demonstration that the decrease in milk yield with advancing lactation is due to a decline in mammary cell number and not cellular activity, it follows that this effect on persistency is due to maintenance of the mammary cell population rather than

maintenance of cellular secretory rate. Indeed, bST appears to maintain cell number in lactating caprine mammary gland (Knight et al., 1990). In experiment 2, the nuclear proliferation antigen Ki-67 was used as an index for the relative proliferation state of mammary tissue. The Ki-67 protein serves as a marker for cells that are engaged in cell cycle progression, due to its presence during all phases of the cell cycle except the quiescent  $G_0$  phase (Gerdes et al., 1984). This protein correlates with cellular proliferation under a variety of physiological conditions in human, rodent, and bovine tissues (Scholzen and Gerdes, 2000; Shayan et al., 1999). Although the precise function of the Ki-67 protein is unknown, it is a member of the family of MPM-2 reactive phosphoproteins that are necessary for control of mitosis, and it is phosphorylated by the cdc2 kinase that is regulated by cyclin B (Endl and Gerdes, 2000a, 2000b). Treatment of first-lactation dairy cows with bST for 7 d increased the proportion of cells expressing the nuclear proliferation antigen threefold (Figure 5;  $P < 0.05$ ). The effect of bST was evident when cows were fed ad libitum (2.5 vs. 0.7%;  $P < 0.01$ ), or when they were restricted to 80% of ad libitum intake (0.8 vs. 0.3%;  $P < 0.05$ ). Cows with limited feed consumption had a lower percentage of mammary cells expressing Ki-67 than did those that were fed ad libitum (1.6 vs. 0.5%;  $P < 0.05$ ). These data strongly support the hypothesis that bST increases cellular proliferation in



**Figure 5.** Effect of bST on mammary cell proliferation and apoptosis. Six cows were fed ad libitum (Ad lib) and six cows were restricted to 80% of their ad libitum intake (Restr). Values shown are for the control period (black bar) and bST treatment period (gray bar) for each dietary group and the overall effect (12 cows). Left panel Ki-67 labeling index, expressed as a percentage of total cells. Right panel: apoptotic index, expressed as a percentage of total cells. Each bar represents the mean  $\pm$  SE for six cows per dietary group and 12 cows for overall effect. Within a dietary category, means without a common superscript differ ( $P < 0.05$ ). Effect of diet on Ki-67 expression was significant ( $P < 0.05$ ). Neither diet nor bST altered the apoptotic index ( $P > 0.05$ ).

the bovine mammary gland and that mammary cell proliferation is blunted by reduced energy balance. Additionally, these data suggest that the tendency for lower mammary cell proliferation during early lactation (14 d) than after peak lactation in experiment 1 may have been due to the influence of reduced energy balance during early lactation.

Although treatment of lactating cows with bST in the present study apparently increased mammary cell proliferation, previous investigations have indicated that bST administration does not result in net growth of the lactating bovine mammary gland (Binelli et al., 1995; Capuco et al., 1989). These previous results do not infer that bST does not increase in the rate of cell proliferation, simply that the rate of cell proliferation does not exceed the rate of cell death. In experiment 2, treatment with bST did not alter apoptotic frequency (Figure 5;  $P > 0.05$ ), and we did not assess true rates of proliferation. We suggest that treatment with bST increased the rate of mammary cell proliferation and in so doing, reduced the rate of mammary regression during lactation.

## CONCLUSION

During early lactation, milk secretion increases to peak due to the continued differentiation of mammary secretory cells and increased secretory activity per cell in the absence of net mammary growth. After peak lactation, the mammary gland undergoes gradual regression through the process of apoptotic cell death. Although cell death exceeds cell proliferation, considerable turnover of mammary cells occurs during lactation. During the course of a bovine lactation, the number of cells formed is approximately 50% of the number originally present in the mammary gland. Consequently, nearly all cells present in the mammary gland at the end of lactation were formed after calving. In contrast, there is very little cell renewal during the shorter lactation of rats. Administration of bST during midlactation increases mammary cell proliferation without a significant effect on apoptosis and thus may account for the ability of bST to increase the persistency of lactation. Schemes that will increase mammary cell proliferation or decrease apoptotic death during lactation will enhance the persistency of lactation. Research focused on the regulation of cell proliferation and apoptosis during lactation will provide information necessary to accomplish this goal.

## REFERENCES

- Anderson, R. R. 1974. Endocrinological control. Pages 97–140 in *Lactation a Comprehensive Treatise*, Vol. I. B. L. Larson and V. R. Smith, eds. Academic Press, New York.
- Anderson, R. R. 1975. Mammary gland growth in sheep. *J. Anim. Sci.* 41:118–123.
- Anderson, R. R., J. R. Harness, A. F. Snead, and M. S. Salah. 1981. Mammary growth pattern in goats during pregnancy and lactation. *J. Dairy Sci.* 64:427–432.
- Banerjee, M. R., and R. J. Walker. 1967. Variable duration of DNA synthesis in mammary gland cells during pregnancy and lactation of C3H/He mouse. *J. Cell Physiol.* 69:133–142.
- Bar-Peled, U., E. Maltz, I. Bruckental, Y. Folman, Y. Kali, H. Gacitua, A. R. Lehrer, C. H. Knight, B. Robinson, H. Voet, and H. Tagari. 1995. Relationship between frequent milking or suckling in early lactation and milk production of high producing dairy cows. *J. Dairy Sci.* 78:2726–2736.
- Bauman, D. E., R. W. Everett, W. H. Weiland, and R. J. Collier. 1999. Production responses to bovine somatotropin in northeast dairy herds. *J. Dairy Sci.* 82:2564–2573.
- Binelli, M., W. K. Vanderkooi, L. T. Chapin, M. J. Vandehaar, J. D. Turner, W. M. Moseley, and H. A. Tucker. 1995. Comparison of growth hormone-releasing factor and somatotropin: Body growth and lactation of primiparous cows. *J. Dairy Sci.* 78:2129–2139.
- Borst, D. W., and W. B. Mahoney. 1980. Diurnal changes in mouse mammary gland DNA synthesis. *J. Exp. Zool.* 214:215–218.
- Bursch, W., S. Paffe, B. Putz, G. Barthel, and R. Schulte-Hermann. 1990. Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. *Carcinogenesis* 11:847–853.
- Capuco, A. V., and R. M. Akers. 1990. Thymidine incorporation by lactating mammary epithelium during compensatory mammary growth in beef cattle. *J. Dairy Sci.* 73:3094–3103.
- Capuco, A. V., R. M. Akers, and J. J. Smith. 1997. Mammary growth in Holstein cows during the dry period: Quantification of nucleic acids and histology. *J. Dairy Sci.* 80:477–487.
- Capuco, A. V., J. E. Keys, and J. J. Smith. 1989. Somatotrophin increases thyroxine-5'-monodeiodonase activity in lactating mammary tissue of the cow. *J. Endocrinol.* 121:205–211.
- Capuco, A. V., D. L. Wood, T. H. Elsasser, S. Kahl, A. Lefcourt, and L. S. Piperova. 2001. Effect of somatotropin on thyroid hormones and cytokines in lactating dairy cows during ad libitum and restricted intake. *J. Dairy Sci.* (in press).
- Concha, C. 1986. Cell types and their immunological functions in bovine mammary tissues and secretions—a review of the literature. *Nord. Vet. Med.* 38:257–272.
- Endl, E., and J. Gerdes. 2000a. Posttranslational modifications of the Ki-67 protein coincide with two major checkpoints during mitosis. *J. Cell Physiol.* 182:371–380.
- Endl, E., and J. Gerdes. 2000b. The Ki-67 protein: Fascinating forms and an unknown function. *Exp. Cell Res.* 257:231–237.
- Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. *J. Dairy Sci.* 79:543–549.
- Garrett, W. M., and H. D. Guthrie. 1998. Detection of bromodeoxyuridine in paraffin-embedded tissue sections using microwave antigen retrieval is dependent on the mode of tissue fixation. *Biochemistry* 1:17–20.
- Gerdes, J., H. B. H. Lemke, H. H. Wacker, U. Schwab, and H. Stein. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* 133:1710–1715.
- Gerdes, J., L. Li, C. Schlueter, M. Duchrow, C. Wohlenberg, C. Gerlach, I. Stahmer, S. Kloth, E. Brandt, and H. D. Flad. 1991. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. *Am. J. Pathol.* 138:867–873.
- Knight, C., and M. Peaker. 1984. Compensatory increases in milk yield after hemimastectomy in lactating goats. *Proc. Physiol. Soc.* 6:36P.
- Knight, C. H., A. H. Docherty, and M. Peaker. 1984. Milk yield in rats in relation to activity and size of the mammary secretory cell population. *J. Dairy Res.* 51:29–35.
- Knight, C. H., P. A. Fowler, and C. J. Wilde. 1990. Galactopoietic and mammogenic effects of long-term treatment with bovine growth

- hormone and thrice daily milking in goats. *J. Endocrinol.* 127:129–138.
- Knight, C. H., and M. Peaker. 1984. Mammary development and regression during lactation in goats in relation to milk secretion. *Q. J. Exp. Physiol.* 69:331–338.
- Labarca, C., and K. Paigen. 1980. A simple, rapid and sensitive DNA assay procedure. *Anal. Biochem.* 102:344–352.
- Miller, R. H., M. J. Paape, and L. A. Fulton. 1991. Variation in milk somatic cells of heifers at first calving. *J. Dairy Sci.* 74:3782–3790.
- National Mastitis Council. 1981. *Procedures for Diagnosis of Bovine Mastitis*. Carter Press, Inc., Ames, IA.
- Pitkow, H. S., R. P. Reece, and G. L. Waszilycsak. 1972. The integrity of mammary alveolar cells in two consecutive lactations. *Proc. Soc. Exp. Biol. Med.* 139:845–850.
- Sakaguchi, K., E. Takeuchi, M. Suzuki, K. Oda, M. Nagino, Y. Nimura, and S. Yoshida. 2000. DNA polymerases and Ki-67 nuclear antigen are induced in correlation with the resected mass of rat liver up to 90%. *Langenbecks Arch. Surg.* 385:135–142.
- Scholzen, T., and J. Gerdes. 2000. The Ki-67 protein: From the known and the unknown. *J. Cell Physiol.* 182:311–322.
- Shayan, P., C. Gerlach, F. U. Hugel, G. Kay, J. D. Campbell, J. Gerdes, and J. S. Ahmed. 1999. The proliferation-associated nuclear protein Ki-67 in the bovine system: Partial characterisation and its application for determination of the proliferation of *Theileria*-infected bovine cells. *Parasitol. Res.* 85:613–620.
- Shi, S. R., M. E. Key, and K. L. Kalra. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* 39:741–748.
- Sordillo, L. M., K. Shafer-Weaver, and D. DeRosa. 1997. Immunobiology of the mammary gland. *J. Dairy Sci.* 80:1851–1865.
- Tucker, H. A. 1981. Physiological control of mammary growth, lactogenesis, and lactation. *J. Dairy Sci.* 64:1403–1421.
- Van Amburgh, M. E., D. M. Galton, D. E. Bauman, and R. W. Everett. 1997. Management and economics of extended calving intervals with use of bovine somatotropin. *Livest. Prod. Sci.* 50:15–28.
- Wilde, C. J., C. V. P. Addey, P. Li, and D. G. Fernig. 1997. Programmed cell death in bovine mammary tissue during lactation and involution. *Exp. Physiol.* 82:943–953.
- Yanai, T., C. Matsumoto, H. Takashima, K. Yoshida, H. Sakai, K. Isowa, T. Iwasaki, Y. Sato, and T. Masegi. 1996. Immunohistochemical demonstration of S-phase cells by anti-bromodeoxyuridine monoclonal antibody in cattle tissues. *J. Comp. Pathol.* 114:265–272.